

## Effect of Temperature on the Growth and Cell Wall Chemistry of a Facultative Thermophilic *Bacillus*

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The morphology and cell wall composition of *Bacillus coagulans*, a facultative thermophile, were examined as a function of growth temperature. The morphology of the organism varied when it was grown at different temperatures; at 37 C the organism grew as individual cells which increased in length with increasing growth temperature. At 55 C it grew in long chains of cells. Cell wall prepared from cells grown at 37 C contained 44% teichoic acid by weight, whereas cells grown at 55 C contained 29% teichoic acid. Teichoic acid from these cells was a polymer of glycerol phosphate containing galactose and ester alanine. The ratio of ester alanine to phosphate was significantly higher in cell walls and teichoic acid from 37 C-grown cells compared with those from 55 C-grown cells. Other differences observed were that cells grown at 55 C contained a lower level of autolytic ability, produced cell walls which bound more  $Mg^{2+}$ , and contained less peptide cross-bridging in its peptidoglycan layer than cells grown at 37 C.

The cell wall composition of gram-positive bacteria has been found to be affected profoundly by changes in environment (7, 14). One of the most significant environmental factors affecting the growth of bacteria is temperature. The effect of temperature on the cell wall chemistry of individual organisms has not been extensively studied because of the limited growth range of most bacteria. Forrester and Wicken (8) reported that the cell walls of two facultative thermophiles, *Bacillus coagulans* and *Bacillus stearothermophilus*, contained an increased proportion of glycopeptides, but less teichoic acids when 55 C-grown cells were compared with those grown at 37 C. Dul and McDonald (5), by employing electron microscopy and antibiotic treatment of *Bacillus subtilis*, postulated that a lack of cell wall cross-linkage might be involved in the increased thermal resistance of cells grown at 55 C. Sutow and Welker (20), however, failed to find any gross differences in the cell wall composition of thermophiles when compared with the cell walls of mesophiles.

The work reported here attempts to correlate growth temperature with the morphology, autolytic activity, and cell wall composition of a facultative thermophile, *B. coagulans*.

### MATERIALS AND METHODS

**Organism.** The bacterial strain (KU) used in this study was a facultative thermophilic bacillus which was isolated from soil. It was identified as *B. coagulans* by the criteria of Gordon and Smith (12). This strain was capable of growth in the temperature range 25 to 56 C.

**Medium and culture conditions.** *B. coagulans* was grown at 37 or 55 C in a tryptone-glucose medium containing 2% tryptone, 0.1% glucose, 0.002%  $MgSO_4 \cdot 7H_2O$ , and 0.001%  $FeCl_3$ . For growth studies the organism was cultured in flasks shaken in a gyratory water bath shaker. For the preparation of cell walls, the organism was grown aerobically in a model F-50 Fermacell fermenter (New Brunswick Scientific Co.) in 40 liters of media with a sparge rate of 2.831  $m^3/s$ . All cultures were harvested in middle to late exponential phase of growth (optical density  $A_{600nm}$  0.7 to 1.0) by quickly transferring the culture into containers filled with chipped ice. A Szent-Gyorgi-Blum continuous-flow attachment on a Sorvall RC-2 refrigerated centrifuge was employed for harvesting the cells.

**Measurement of growth rate constant.** The growth rate constant,  $k$ , was calculated from the relationship  $Mk = dM/dt$ , where  $M$  represents dry cell mass and  $t$  represents time. Cultures of *B. coagulans* were grown in 500 ml Erlenmeyer flasks containing 150 ml of media. They were monitored spectrophotometrically at  $A_{600nm}$ , and the values obtained were converted to mass units by using a calibration curve which was obtained for cells grown at each temperature. The  $\ln M$  value was plotted against time, the slope of this plot being equal to the growth rate constant from the relationship  $\ln M_t/M_0$ .

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$= k(t - t_0)$ , where  $M_0$  and  $M_t$  are dry cell mass units at time  $t_0$  and after time  $t$  of growth, respectively.

**Phase microscopy.** Bacteria which had been concentrated by centrifugation were immobilized between a glass cover slip and a microscope slide coated with a thin layer of 1% Noble agar before being photographed.

**Electron microscopy.** An RCA EMU-s electron microscope, operating at 50 kV, was used in this study. Bacteria for thin-section observation were prepared as described by Dul and McDonald (5). Thin sections of the Epon-embedded bacteria were obtained with a Porter-Blum model 1 ultramicrotome employing glass knives. Sections were picked up on copper grids and were double stained with uranyl acetate and lead citrate. Specimens for negative-stain observation were prepared by placing a drop of aqueous suspension of the specimen on a carbon-coated grid. The drop was drawn off after 1 min, and immediately a drop of stain, either 1% uranyl acetate, pH 4.5, or 0.5% uranyl oxalate, pH 6.5, was placed in the grid and drawn off after 20 s. The grid was air-dried prior to observation.

**Preparation and hydrolysis of cell walls.** Cell walls were prepared by the method of Sutow and Welker (20). Purified cell walls were lyophilized and stored in a vacuum desiccator over potassium hydroxide pellets and phosphorus pentoxide. For hydrolysis, 2.5 mg of lyophilized cell walls were suspended in 1 ml of 6 N HCl and placed in hydrolysis tubes which were evacuated and sealed. The samples were heated at 100 C for 4, 8, 16, and 24 h. After hydrolysis, the tubes were opened, and the hydrolysates dried in vacuo. Because amino sugars decompose rapidly during the hydrolysis procedure, the values obtained for the various hydrolysis times were extrapolated to zero time to approximate the actual amounts of the amino sugars.

**Treatment of cell walls with  $MgCl_2$ .** Cell walls were prepared for magnesium binding as described by Heptinstall et al. (13). Samples of lyophilized cell wall (25 mg), from which all divalent cations were removed, were suspended in 10 ml of 50 mM sodium acetate buffer, pH 5.0, containing 0.25 mmol of  $MgCl_2$ . This suspension was incubated at 25 C for 16 h, then centrifuged at  $17,000 \times g$  to pellet the cell walls, washed four times with deionized water, and lyophilized.

**Extraction and hydrolysis of teichoic acid.** Teichoic acid was extracted from purified cell walls by the method of Forrester and Wicken (8). The teichoic acid fraction and resulting cell wall residue were dried in vacuo and weighed. For hydrolysis, 2 mg of teichoic acid fraction was heated at 100 C with 2 N HCl (0.2 ml) in sealed, evacuated hydrolysis tubes for 3 h. After hydrolysis, the tubes were opened, and the hydrolysates dried in vacuo.

**Analytical methods.** Amino acid and amino sugar analyses were performed with a Beckman model 120 C amino acid analyzer. To separate all of the components, a modification of the method of Bensen et al. (3) was used. A 57-cm column, packed with Beckman custom resin (UR 30,  $Li^+$  form), was used for separating neutral and acidic amino acids and

amino sugars. The buffer systems used are listed in Table 1. The dry-sample hydrolysates were taken up in 3 ml of diluter buffer and applied to the column. A buffer flow rate of 68 ml/h, a ninhydrin flow rate of 34 ml/h, and a column temperature of 37 C were maintained throughout the analysis. A buffer change from  $E_1$  to  $E_2$  was made at 80 min, prior to aspartate elution. The cell wall components were separated into a sequence as aspartic acid, serine, muramic acid, glutamic acid, glycine, alanine, valine, glucosamine, galactosamine, and diaminopimelic acid. The total analysis time for neutral and acidic compounds in each sample was 300 min. The basic cell wall components, lysine and ammonia, were separated on an 8-cm column packed with Beckman custom spherical resin PA 35,  $Na^+$  form. A pH 5.25 sodium citrate buffer with the following composition was used as an elution buffer: sodium citrate, 137.26 g; 12 N HCl, 26 ml; Brij 35 solution (50 g of Brij 35 in 100 ml of distilled water), 0.8 ml; and octanoic acid, 0.03 ml (made up to 4 liters with distilled water).

Phosphate was determined by the method of Ames (1). Alanine in teichoic acid hydrolysates was determined by the method of Rosen (16). Polyalcohol and carbohydrate components of teichoic acid hydrolysates were determined by gas-liquid chromatography of their trimethylsilyl (TMS) derivatives by using a Varian aerograph model 1740 gas-liquid chromatograph equipped with a hydrogen flame detector. Determinations were made by using a column (0.3 by 152 cm) packed with 3% SE-30 methylsilicone on Varaport 30, 100 to 200 mesh. The chromatograph was operated on a linear temperature program at a rate of 2 C/min from 120 to 170 C with a carrier gas ( $N_2$ ) flow rate of 25 ml/min. Prior to derivative formation with TRI SYL "Z" (Pierce Chemical Co, Rockford, Ill.), the teichoic acid hydrolysates were treated with orthophosphoric monoester phosphohydrolase (EC 3.1.3.1) (22). Anhydrous glycerol was prepared as described by Vogel (21). TMS derivatives of anhydrous glycerol and galactitol were prepared using TRI SYL "Z". Standard TMS D-galactose was obtained from Pierce Chemical Co., Rockford, Ill. Glycerol was also determined by using glycerol dehydrogenase (EC 1.1.1.6) (4). Galactose in teichoic acid hydrolysates was reduced to galactitol with sodium borohydride (19). Free amino groups of diaminopimelic acid in intact cell walls were assayed by the method of

TABLE 1. Composition of lithium citrate buffers used in the amino acid analysis<sup>a</sup>

Component	Sample diluter (pH 2.2)	Buffer $E_1$ (pH 2.6 $\pm$ 0.01)	Buffer $E_2$ (pH 3.25 $\pm$ 0.01)
Lithium citrate 4 H <sub>2</sub> O (g)	14.1	60.2	60.2
Lithium chloride (g) . . . .		23.8	23.8
Thioglycol (ml) . . . . .	10.0	10.0	10.0
Concentrated HCl (ml) . .	13.0	47.0	36.4

<sup>a</sup> The final volume for the three buffer systems was (in liters): sample diluter, 0.5; buffer  $E_1$ , 4.0; and buffer  $E_2$ , 4.0.

Ghuysen et al. (11). Magnesium was determined in  $MgCl_2$ -treated cell walls after wet digestion (6) by using the spectrophotofluorometric assay as described by Schacter (17). An Aminco-Bowman spectrophotofluorometer was used in this assay.

**Autolysis of cell suspensions.** Autolysis of whole cells was observed by recording the decrease in optical density at  $A_{600nm}$  of cells suspended in 0.01 M phosphate buffer at pH 8.5. Suspensions were incubated at either 37 or 55 C and the  $A_{600nm}$  was recorded at various intervals.

## RESULTS

**Cell morphology.** At 37 C, *B. coagulans* appeared by phase contrast microscopy as single and paired cells (Fig. 1A). As the growth

temperature was increased to 45 C and 50 C, cells became longer (Fig. 1B, C). At the 55 C growth temperature, the organism occurred as long chains of cells (Fig. 1D). Electron microscope examination of thin sections of cells grown at 55 C revealed rod-shaped cells linked end to end to form chains (Fig. 2B). Newly initiated septa were frequently seen in these preparations. In addition, the cell wall of the cells grown at 55 C appeared thicker and had a rougher-appearing outer surface than did the walls of cells grown at 37 C (Fig. 2A, B).

**Cell growth.** Cells grown at 55 C (under identical conditions) had a higher-growth-rate constant than did cells grown at 37 C (Table 2).

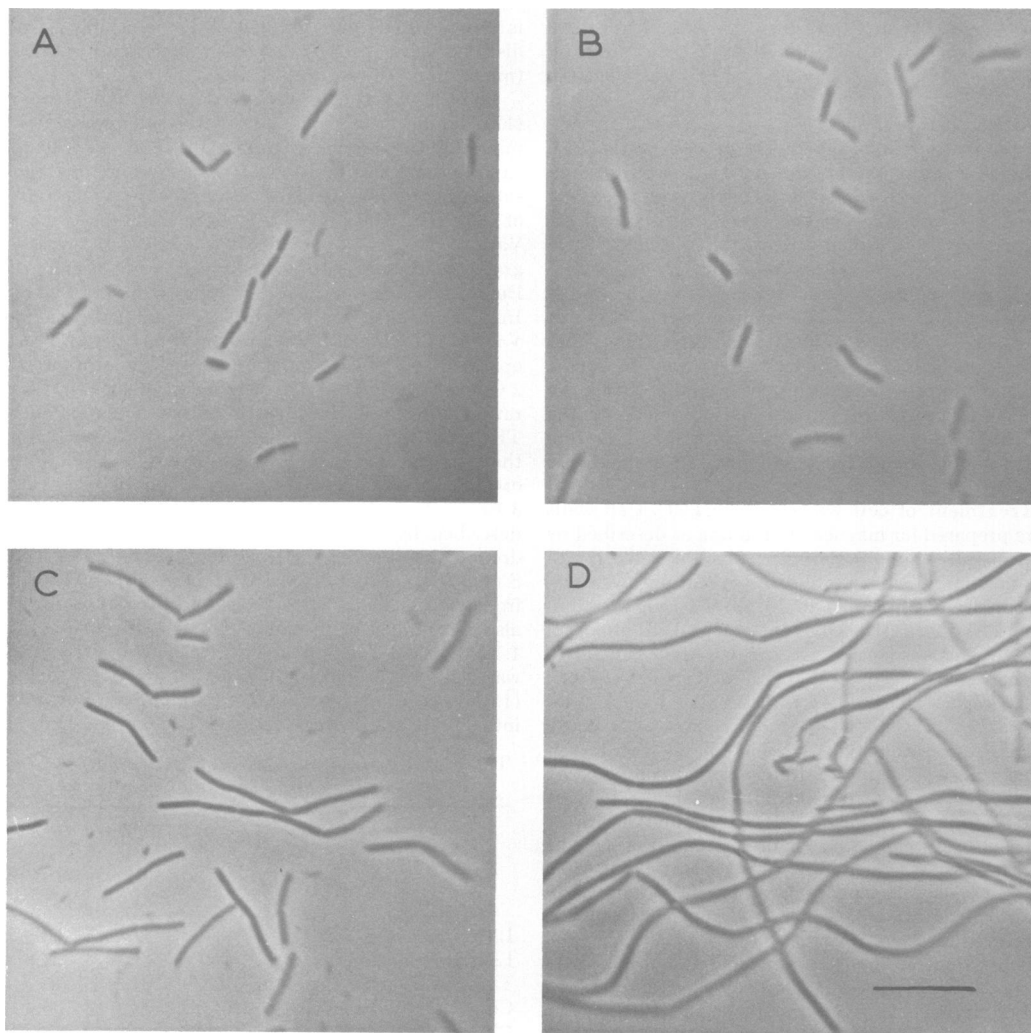


FIG. 1. Phase contrast micrographs of *B. coagulans* during the exponential growth phase. Cells were grown at: A, 37 C; B, 45 C; C, 50 C; and D, 55 C. Bar represents 1.0  $\mu m$ .

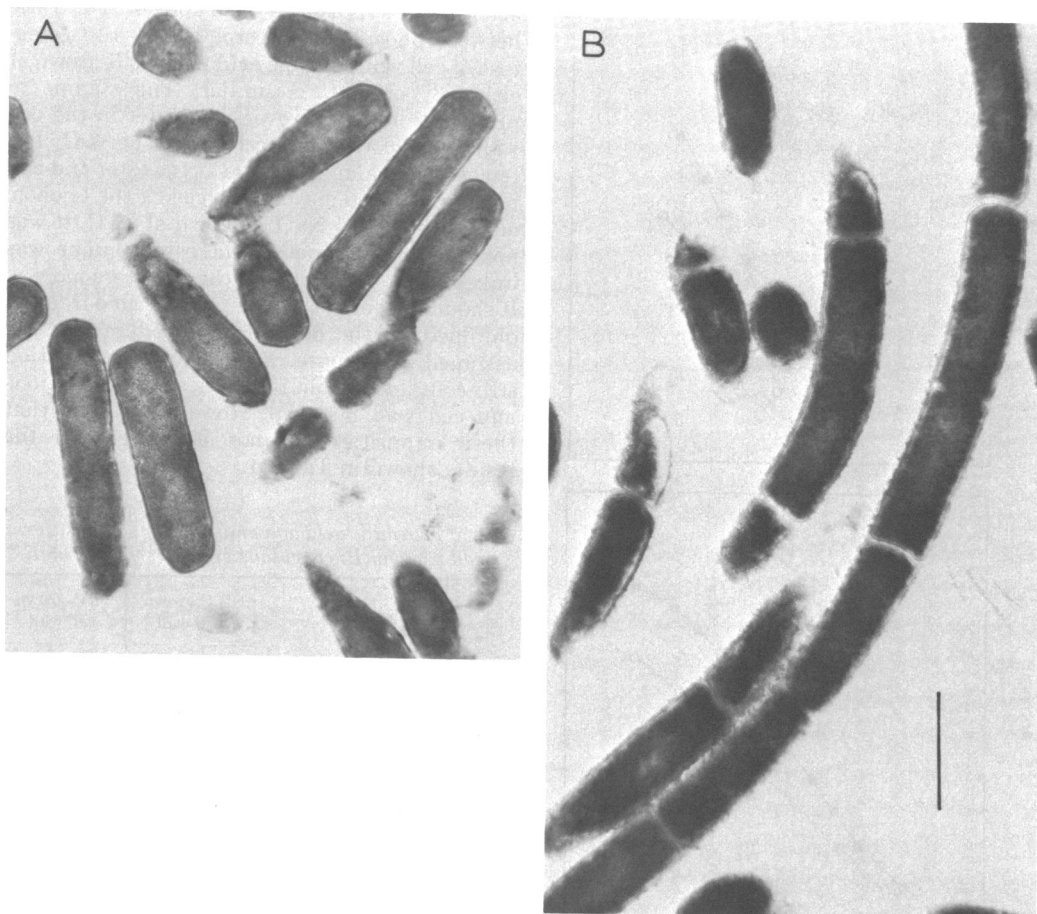


FIG. 2. Electron micrographs of *B. coagulans* during the exponential growth phase. A, Cells grown at 37 C; B, cells occurring in long chains and grown at 55 C. Bar represents 1.0  $\mu\text{m}$ .

The growth rate constant was determined from the slope of the plots shown in Fig. 3. The generation time at each growth temperature was calculated from the growth rate constant and not from viable count data. Viable counts were considered invalid with the 55 C-grown cells because of chaining, and they were irreproducible with the 37 C-grown cells because of clumping.

TABLE 2. Growth rate constants and generation times of *B. coagulans* grown at 37 and 55 C with shaking<sup>a</sup>

Growth temp	Shaking speed			
	180 rpm		260 rpm	
	<i>k</i>	G (min)	<i>k</i>	G (min)
37 C	0.0245	28	0.0257	27
55 C	0.0295	23	0.0308	23

<sup>a</sup>*k*, Growth rate constant; G, generation time.

**Wall morphology.** Cell walls were prepared from cells grown at 37 and 55 C and examined by electron microscopy. Cell walls from cells of each growth temperature appeared flattened and retained a rod shape. The surface of the cell wall from 55 C-grown cells (Fig. 4B) had a rougher appearance than the cell wall of the 37 C-grown cells (Fig. 4A). Cell walls of the 37 C-grown cells stained at an optimal pH of 4.5 to 5.5 with 1% uranyl acetate, whereas cell walls of the 55 C-grown cells stained optimally at pH 6.0 to 6.8 with 0.5% uranyl oxylate.

**Wall chemistry.** Purified walls from cells grown at both 37 and 55 C contained glucosamine and muramic acid as their major amino sugar components, and alanine, glutamic acid, and diaminopimelic acid as their major amino acid. Serine, glycine, valine, and lysine were also found. The cell walls from the 55 C-grown cells contained a greater amount of glucosamine, muramic acid, and glutamic acid than

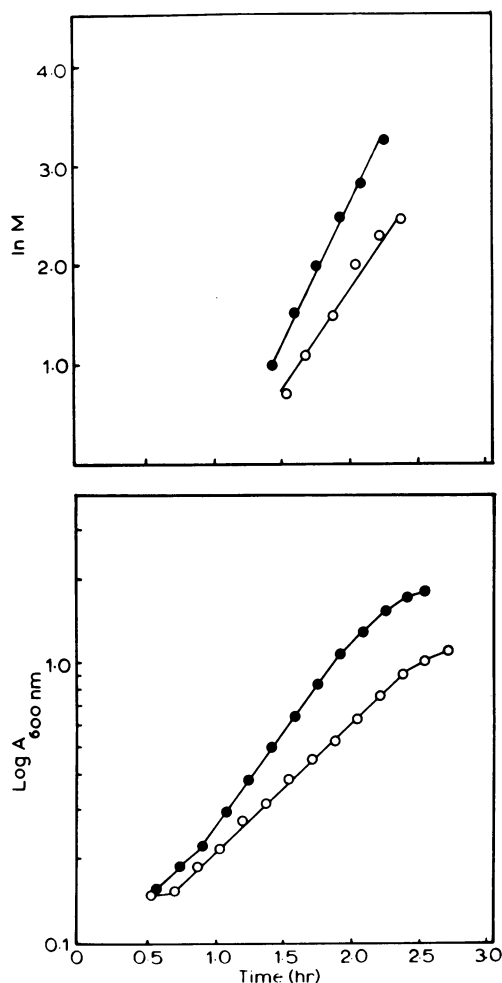


FIG. 3. *B. coagulans* grown at 37 C (O) and 55 C (●) as measured by increase in cell dry mass (top) and  $A_{600nm}$  (bottom). Shaking speed for flasks was 260 rpm.

did the cell walls from the 37 C-grown cells (Table 3).

The ratio of free epsilon amino groups of diaminopimelic acid to diaminopimelic acid residues in the cell wall of 37 C-grown cells was 0.52, whereas that of the wall of 55 C-grown cells was 0.69. This ratio indicates the proportion of diaminopimelic acid residues not involved in peptide cross-bridging present in the cell wall. By using this conversion, an amount of cross-bridging of 48 and 31% was found for cell walls from 37 C-grown and 55 C-grown cells, respectively.

Trichloroacetic acid-extractable teichoic acid accounted for 44 and 29% of the dry weight of the cell wall of 37 C- and 55 C-grown cells, respectively. The composition of the teichoic

acid fraction is shown in Table 4. The ratio between phosphate, glycerol, and D-(+)-galactose of cell wall teichoic acid from cells grown at either 37 or 55 C is similar. The alanine to phosphate ratio, however, is greater in the cell wall teichoic acid from cells grown at 37 C. The teichoic acid alanine occurs at the ester and can be completely removed by treating the teichoic acid with 0.01 N NaOH for 1 h at 37 C. It was not determined whether the ester alanine was linked to the glycerol or to the galactose moiety. It should be noted that the teichoic acid fraction obtained by the described procedure was not assumed to be pure. Presumably this fraction still contained some impurities such as cations and cell wall material. However, we feel that these impurities did not interfere with the results shown in Table 4.

TABLE 3. Amino acid and amino sugar composition of cell walls of *B. coagulans* grown at 37 and 55 C

Component	37 C-grown cell wall		55 C-grown cell wall	
	Amt <sup>a</sup>	Molar ratio <sup>b</sup>	Amt <sup>a</sup>	Molar ratio <sup>b</sup>
Glucosamine	390	1.46	483	1.31
Muramic acid	320	1.19	480	1.30
Galactosamine	90	0.34	54	0.15
Total hexosamines	800		1,017	
Alanine	1,190	4.44	1,090	2.96
Glutamic acid	268	1.00	368	1.00
Diaminopimelic acid	338	1.26	359	0.98
Aspartic acid	2		2	
Serine	33		41	
Glycine	7		7	
Valine	9		9	
Lysine	20		20	

<sup>a</sup> Values are expressed as nanomoles per milligram of cell wall.

<sup>b</sup> Based on glutamic acid arbitrarily designated as 1.00.

TABLE 4. Composition of trichloroacetic acid-extractable teichoic acid fraction from cell walls of *B. coagulans* grown at 37 and 55 C

Component	37 C-grown cell wall teichoic acid		55 C-grown cell wall teichoic acid	
	Amt <sup>a</sup>	Molar ratio	Amt <sup>a</sup>	Molar ratio
Phosphate	0.97	1.00	1.67	1.00
Glycerol	0.93	0.96	1.66	0.99
D-(+)-galactose	0.27	0.28	0.44	0.27
Alanine ester	1.05	1.08	0.43	0.26

<sup>a</sup> Values are expressed as micromoles per milligram of teichoic acid.

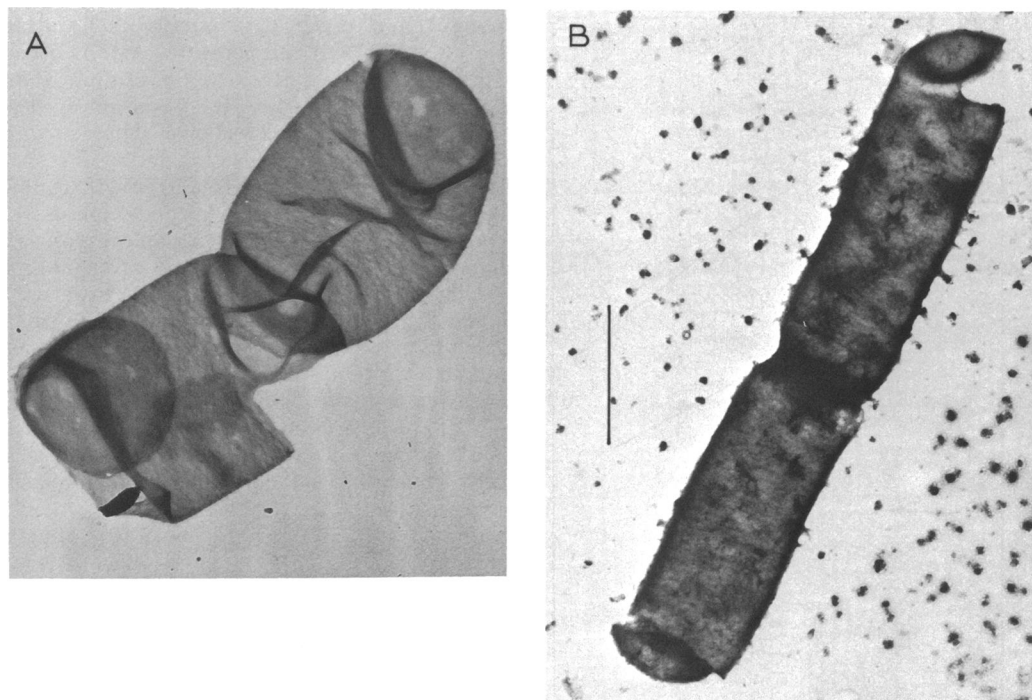


FIG. 4. Electron micrographs of negatively stained preparations of *B. coagulans* cell walls. A, Cells grown at 37 C and stained with 1% uranyl acetate, pH 4.5; B, cells grown at 55 C and stained with 0.5% uranyl oxylate, pH 6.5. Bar represents 1.0  $\mu$ m.

**Magnesium binding.** The alanine to phosphate ratio can also be correlated with the amount of magnesium per phosphate residue the cell wall can bind (Table 5). The 55 C-grown cell wall, having a low alanine to phosphate ratio, binds more magnesium than the cell wall from 37 C-grown cells which have a higher alanine to phosphate ratio.

**Autolytic activity.** Whole-cell suspensions of 37 C- and 55 C-grown cells incubated at either 37 or 55 C showed optimal autolytic activity in phosphate buffer at pH 8.5. Suspensions of 37 C-grown cells had a greater autolytic activity at both the 37 and 55 C incubation temperatures than did suspensions of 55 C-grown cells

(Fig. 5). Both 37 C- and 55 C-grown cell suspensions exhibited linear autolytic responses if incubated at 37 C when plotted as  $\log A_{600nm}$  versus time. However, 37 C- and 55 C-grown cell suspensions incubated at 55 C exhibited nonlinear autolytic responses when plotted in a similar manner.

## DISCUSSION

*B. coagulans* (KU) undergoes morphological changes when grown at different temperatures; individual cells become larger and eventually occur in chains as the growth temperature is increased. Accompanying the change in morphology is a change in its cell wall composition. Cells grown at 55 C contained less teichoic acid per milligram of cell wall than did cells grown at 37 C. The amount of teichoic acid present in bacterial cell walls has been shown to vary among species (2) and within individual species grown under limiting phosphate or magnesium concentration, or under other cultural conditions including temperature (7, 8). The teichoic acid of *B. coagulans* consists of glycerol, phosphate, galactose, and ester alanine. The most significant difference between the teichoic acid extracted from 37 C- and 55 C-grown cells was the ester alanine to phosphate ratio. The

TABLE 5. Relationship of the ester alanine to phosphate ratio to the magnesium binding capacity of the cell walls of *B. coagulans* grown at 37 and 55 C

Cell wall prepn	Ester alanine content of cell walls <sup>a</sup>	Magnesium content of cell walls <sup>b</sup>
37 C-grown cells	0.90	0.30
55 C-grown cells	0.34	0.53

<sup>a</sup> Values are expressed as moles of ester alanine per mole of phosphate.

<sup>b</sup> Values are expressed as equivalents of magnesium per mole of phosphate.

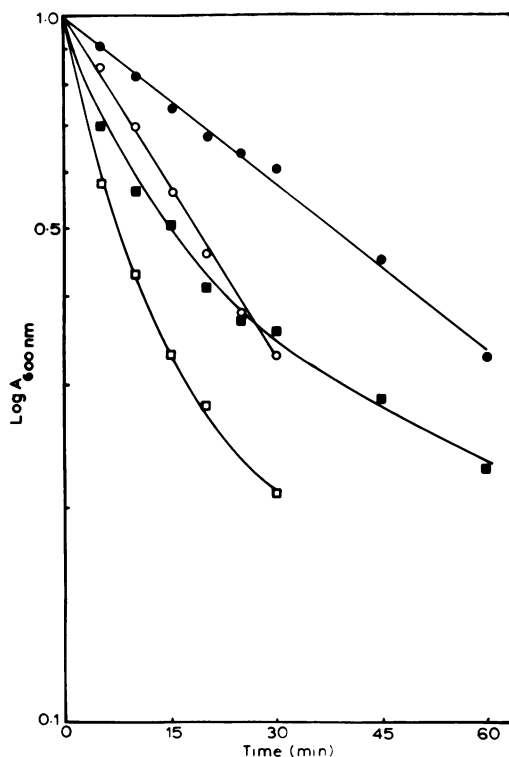


FIG. 5. Autolysis of cell suspensions of *B. coagulans* at 37 and 55 C. Organism grown at 37 C and incubated at 37 C (○) or incubated at 55 C (□). Organism grown at 55 C and incubated at 37 C (●) or incubated at 55 C (■).

amount of esterified alanine in teichoic acid has been shown to vary with the growth medium, and its probable function is in controlling the charge on the cell surface (13). It was also shown that the ester alanine to phosphate ratio is related to the ion-binding capacity of the cell surface (13). *B. coagulans* (KU), grown at 37 C and containing a higher ester alanine to phosphate ratio, would be expected to have a more neutral net charge than cells grown at 55 C. The charge difference may explain the clumping observed with cells grown at 37 C, but not with 55 C-grown cells. The charge difference also may account for the fact that the cell walls isolated from 37 C-grown cells did not bind as much  $Mg^{2+}$  as did those from 55 C cells. Cell walls of *Staphylococcus aureus* H having ester alanine to phosphate ratios of 0.60 and 0.20, when grown under different conditions, bound 0.23 and 0.51 equivalents of  $Mg^{2+}$  per mol of phosphate, respectively (13). This agrees closely with the  $Mg^{2+}$ -binding data obtained in this study for 37 C- and 55 C-grown cells.

Changes were also observed in the peptidogly-

can portion of the *B. coagulans* KU cell wall in relation to growth temperature. At 55 C, cell walls contained less peptide cross-bridging than did walls from 37 C cells. Variation in the extent of cross-bridging has been shown to vary among species (15) and within individual species when subjected to altered growth conditions (14, 18). Accompanying the temperature-related morphological and cell wall composition changes was a decrease in the autolytic ability of cells grown at 55 C. Forsberg and Rogers (9) reported that the autolytic activity of *Bacillus licheniformis* mutants could be reduced without significant alteration of the growth rate. They suggested that the changes in autolytic activity were due to its altered wall composition and not to a decrease in autolysin(s) content. In this study, the observed nonlinearity of autolysis plots with cell suspensions of *B. coagulans* KU incubated at 55 C, regardless of its growth temperature (Fig. 5), indicates that thermal inactivation of one or more autolysins occurred. The explanation for chain formation and decreased autolytic activity of cells grown at 55 C may involve both a change in the synthesis of autolysins and a decrease in the amount of these enzymes because of thermal inactivation.

The question whether the changes observed in the cell wall teichoic acid and peptidoglycan as a function of growth temperature are in any way responsible for the ability of *B. coagulans* KU to grow at 55 C or are secondary changes brought about by growth at the elevated temperature is not possible to answer at this time. It is conceivable that the observed results are due to a lower activity of the enzymes responsible for adding ester alanine to the teichoic acid and cross-bridging in the peptidoglycan at 55 C. For example, although proteins from obligate thermophiles are inherently quite thermostable, we observed that some selected glycolytic enzymes from *B. coagulans* KU grown at 55 C were as heat labile as enzymes isolated from mesophilic organisms (unpublished data). Thus, the abnormal thermostability of proteins cannot be a contributing factor in the ability of this facultative thermophile to grow at 55 C. The obvious question arises as to how an organism can grow at an elevated temperature if its key enzymes are rapidly inactivated. One possibility is that a rapid resynthesis of proteins is occurring. The ability of the protein-synthesizing machinery to function at 55 C may be related to the increased  $Mg^{2+}$ -binding capacity of the cell wall at this temperature. Friedman and Weinstein (10) have shown that in vitro amino acid incorporation by *B. stearothermophilus* components required a higher  $Mg^{2+}$  concentration for poly(U)-

directed phenylalanine incorporation at 65 C when compared with the same system at 37 C. We are continuing these studies to determine the effect of temperature on protein synthesis by *B. coagulans*.

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